EXHIBIT A

Annu. Rev. Cell Dev. Biol. 1998. 14:197–230

BIOLUMINESCENCE

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KEY WORDS: luciferase, autoinducer, quorum sensing, GFP, symbiosis

ABSTRACT

Bioluminescence has evolved independently many times; thus the responsible genes are unrelated in bacteria, unicellular algae, coelenterates, beetles, fishes, and others. Chemically, all involve exergonic reactions of molecular oxygen with different substrates (luciferins) and enzymes (luciferases), resulting in photons of visible light (≈50 kcal). In addition to the structure of luciferan, several factors determine the color of the emissions, such as the amino acid sequence of the luciferase (as in beetles, for example) or the presence of accessory proteins, notably GFP, discovered in coelenterates and now used as a reporter of gene expression and a cellular marker. The mechanisms used to control the intensity and kinetics of luminescence, often emitted as flashes, also vary. Bioluminescence is credited with the discovery of how some bacteria, luminous or not, sense their density and regulate specific genes by chemical communication, as in the fascinating example of symbiosis between luminous bacteria and squid.

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1081-0706/98/1115-0197$08.00

INTRODUCTION

Basic research on bioluminescence, often carried out for the pleasure of uncovering how organisms manage the feat of converting chemical energy into light, can now be credited with remarkable advances in fields unrelated to bioluminescence. An important example stems from the study of bacterial luminescence, where the discovery of autoinduction led to the notion of "quorum sensing," and thus of chemical communication between bacteria, which is throwing light on processes ranging from pathogenesis to symbiosis. Another example is the green fluorescent protein (GFP), discovered decades ago as an accessory emitter in coelenterate bioluminescence and now used extensively as a reporter protein.

Throughout evolution, bioluminescence has been reinvented many times; some 30 different independent systems are still extant. The enzymes (luciferases) catalyzing the light-emitting reactions of fireflies, coelenterates, and bacteria, for example, show no homology to each other, and the substrates (luciferins) of these reactions are unrelated chemically. There is, however, one common thread tying together different systems at the molecular level: All bioluminescences (with fungal luminescence a possible exception) are luciferase-catalyzed reactions of molecular oxygen with the luciferins. All involve a luciferase-bound peroxy-luciferin intermediate, the breakdown of which provides energy for excitation.[1]

Several factors affect the color of a bioluminescence. In the simplest case, the emission matches the fluorescence of an excited luciferase-bound product of the reaction. The luciferase structure can itself alter the color, as in the firefly, where single amino acid substitutions in the luciferase result in significant shifts in the emission spectrum. In bacteria and coelenterates, the chromophores of accessory proteins associated with a luciferase may serve as alternate emitters, such as the yellow fluorescent protein (YFP) in bacteria and GFP in coelenterates.

[1]The terms luciferin and luciferase are generic, referring to the substrate and enzyme in a bioluminescent reaction irrespective of their structures, so they must be qualified by specifying the organism. Luciferase genes from different groups of organisms are typically not homologous and may be distinguished by their different abbreviations: *lux* (bacterial), *luc* (firefly), and *lcf* (dinoflagellate).

The cell biology and regulation of bioluminescence differ among groups. While bacteria and some other systems emit light continuously, in many the luminescence occurs as flashes, typically of 0.1–1 s duration. These require a rapid turn on and off of an enzymatic reaction, with reagents sequestered appropriately and subject to quick mobilization. Luminescent organelles in scale worms and fireflies represent modifications of endoplasmic reticulum and peroxisomes, respectively. Dinoflagellate organelles (scintillons) are novel cytoplasmic structures whose flash is triggered by a rapid pH change within the organelle. In coelenterates, flashing is caused by calcium entry, and the calcium sites on the relevant proteins have homologies with calmodulin, whereas in fireflies, the triggering agent is unknown, although oxygen is a candidate.

Progress in the fundamental knowledge of bioluminescence has also led to numerous gene-reporting techniques and very sensitive analytical methods; these are not reviewed here, even though thanks to them new basic knowledge is now acquired in many areas of biology and medicine.

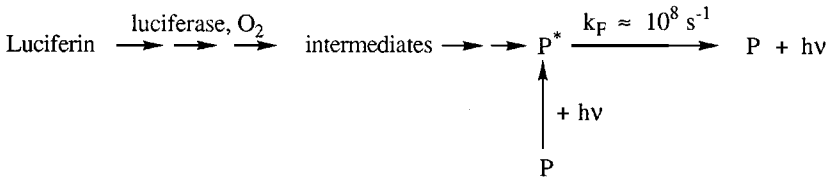
Work on bioluminescence is actively pursued at all levels, from the perspective of the naturalist to that of the photochemist. Although the last Annual Review article on this subject dates back thirty years (Hastings 1968), many aspects of bioluminescence have been the objects of selective reviews in recent years, and these are cited here. The present account combines a broad overview of the chemistry and cellular control of bioluminescence with deeper discussions of selected topics, in the hope of conveying the interest and importance of bioluminescence to a reader unfamiliar with the field.

DIVERSITY OF CHEMISTRIES

How Is Light Generated?

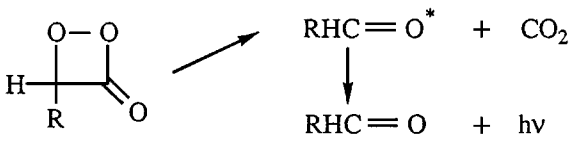
Each time a photon of visible light is emitted at room temperature, by a living organism or chemicals in a test tube, the reaction responsible for the creation of the excited state, and thus for the emission, must be a very exergonic process, because a photon of green light ($\approx$500 nm), for example, corresponds to $\approx$60 kcal per mole (or about eight times the energy released by the hydrolysis of ATP to ADP). It must also happen in one step because the pooling of the energy of two or more exothermic reaction steps can occur only in rare conditions not encountered here. The actual emission of chemiluminescence or bioluminescence (which is a chemiluminescence that requires an enzyme) is the extremely rapid final process of a usually multistep reaction, in which the penultimate step is the generation of a molecule in an electronically excited state, P^*. The excited state of the emitter has a very short lifetime. It holds the reaction energy for no more than a few nanoseconds before releasing it in the form of a photon, and it cannot be distinguished from the excited singlet state (thus fluorescence)

created by the absorption of a photon by P, as shown by a *vertical arrow* below (for example, see Turro 1978; for a brief review, see Wilson 1995).



Therefore, in principle, the spectrum of a bio- or chemiluminescence matches the fluorescence spectrum of the reaction product P, and this should provide a clue as to the identity of this product. As a corollary, there cannot be an efficient bioluminescence if the emitter has a low fluorescence efficiency. In an in vitro luciferase/luciferin reaction, kinetic events happening on a time scale longer than nanoseconds reflect processes preceding the formation of the excited emitter, not its very fast radiative deactivation.

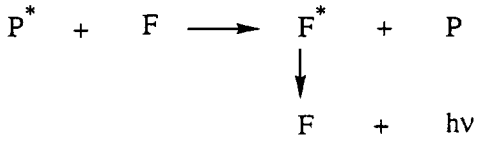
Among the simplest model systems studied chemically, the decomposition of four-membered ring dioxetanones are especially relevant to bioluminescence (Shimomura 1982). These strained and energy-rich peroxides decompose to form CO_2 and an often-excited carbonyl compound, releasing a large reaction energy at the small initial cost of breaking the comparatively weak O-O bond.



The cleavage of a dioxetanone, formed in the oxidation of a luciferin, may be catalyzed by an internal electron transfer to the O-O bond from the R moiety if its oxidation potential is appropriately low; the consequence is a fast cascade of processes terminating in the generation of the excited state of RHC=O, possibly via a final charge-annihilation process. Though yet unproven, even in model systems, this proposal of a chemically induced electron-exchange luminescence, or CIEEL, is often invoked and discussed in the bioluminescence literature, notably in the firefly and coelenterate cases, as well as in the bacterial luciferase reaction, which does not involve a dioxetanone intermediate (see below) (Schuster 1979, Catalani & Wilson 1989, McCapra 1997).

The expectation that the bioluminescence should match spectrally the fluorescence of the reaction product may not be realized for several reasons. One is

that the emission may originate from an enzyme-bound intermediate or product; the intensity and spectrum may then differ from that of the free excited product. The firefly system best illustrates this case. Another possibility is that a second fluorophore (F, scheme below) such as GFP is present, to which the energy carried by the primary excited species (P^*) is transferred, thereby causing this accessory fluorophore to become excited and emit its own fluorescence.



Electronic energy can, in fact, be transferred from one molecule to another in several ways (Lamola 1969, Turro 1978). One way is trivial; the accessory fluorophore simply absorbs the light emitted by the primary excited state and re-emits it as its own fluorescence. In contrast to this radiative process, radiationless energy transfer can happen by either of two mechanisms. If there is very close contact between energy donor and acceptor, electrons can jump between orbitals on donor and acceptor, resulting in so-called electron-exchange (or Dexter) energy transfer. Alternatively, if there is a good overlap between the absorption spectrum of the second fluorophore and the emission spectrum of the primary emitter, energy transfer can take place at distances much greater than molecular diameters, via so-called Förster resonance energy transfer (FRET) (Wu & Brand 1994). The rate is an inverse function of r^6, where r is the distance between donor and acceptor, and strongly depends on their mutual orientation. In favorable situations, resonance transfer remains efficient over tens of Å. The green emission of coelenterates is considered to be the result of radiationless energy transfer to GFP (see below).

In still another situation, an accessory fluorophore may actually participate in the reaction, by binding to an enzyme-substrate intermediate and influencing the kinetics of the reaction. The fluorophore, then, may acquire its excitation energy in a different way, i.e. bypassing excitation of the primary emitter. The bacterial accessory emitters (YFP and LumP) may function in this way (see below). The complexities of this aspect of bioluminescence at the mechanistic and molecular levels will be obvious in the discussion of specific examples below.

Bacteria

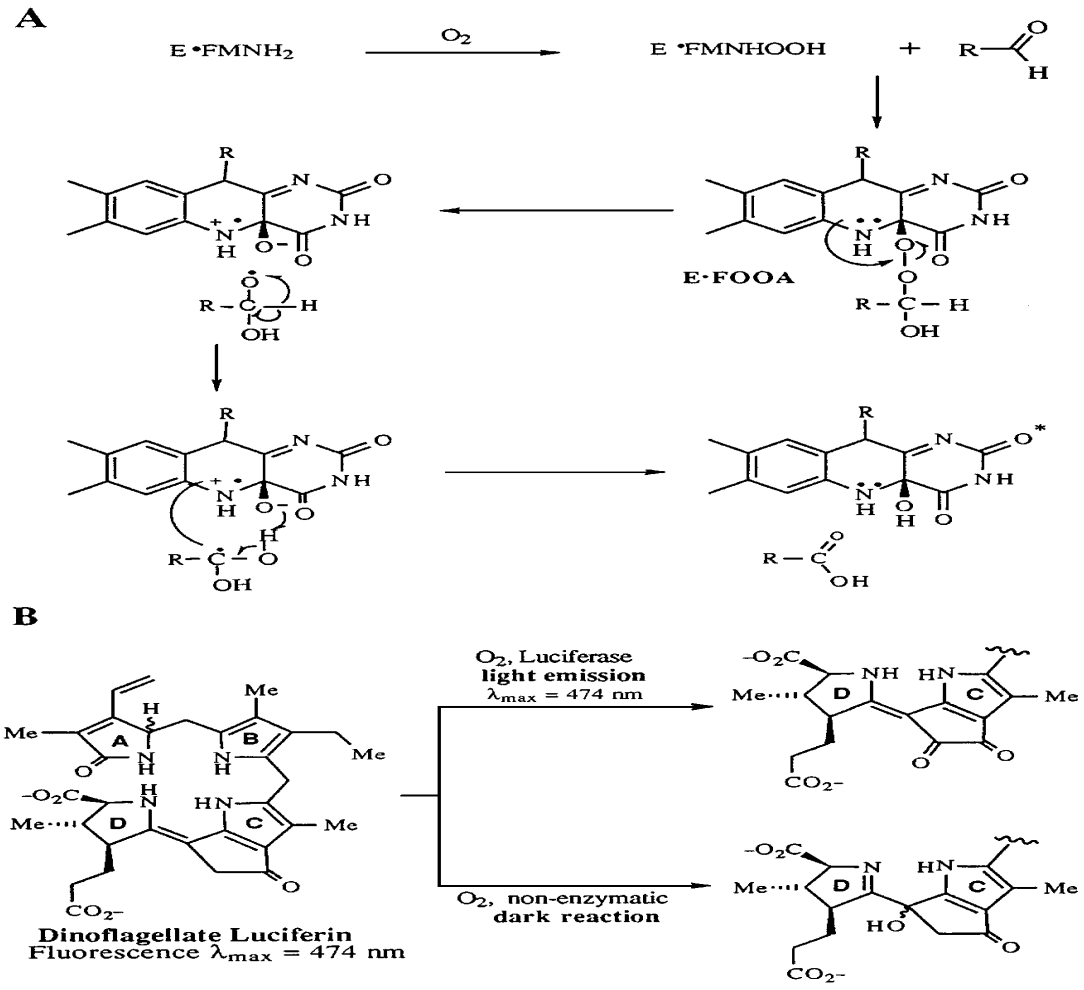
Bacterial bioluminescence is the foremost example of a bioluminescence that does not appear to involve the intermediacy of a dioxetanone. Luciferase catalyses a mixed function oxidation of a long chain aldehyde and reduced flavin

mononucleotide, $FMNH_2$ (Lee et al 1991, Baldwin & Ziegler 1992, Meighen 1994, Tu & Mager 1995). Once bound to the enzyme, $FMNH_2$ is protected against autoxidation, which is faster than the enzymatic reaction. The first step of the catalytic reaction is the formation of an unusually stable, chromatographically purifiable, luciferase-bound flavin hydroperoxide (Hastings et al 1973, Vervoort et al 1986). In a second step, aldehyde reacts to form a postulated peroxyhemiacetal, E · FOOA, spectrally tracked but not yet isolated (Macheroux et al 1993). In spite of indications that there are many steps involved (Abu-Soud et al 1993), it is the lifetime of E · FOOA that determines the rate of the reaction. The emitter is the enzyme-bound 4a-hydroxyflavin, identified by its emission spectrum (Kurfürst et al 1987). The quantum yield is $\approx$0.3 hν per reacting $FMNH_2$ molecule (Figure 1).

Electrochemical studies (Mager & Tu 1995) and the correlation of the reaction rate with the oxidation potentials of substituents in the 8-position (Eckstein et al 1993) suggest that the rate-determining step is an electron transfer (or charge redistribution) from the dihydroflavin moiety to the especially weak peroxide bond within the peroxyhemiacetal, thereby causing the O-O bond cleavage. The excitation step is regarded as a charge annihilation between two radical-ion centers, occurring within the constraints of the enzyme pocket. Since none of these postulated reaction intermediates is stable apart from the enzyme, the mechanism remains perforce hypothetical. Up to now, the chemiluminescence efficiencies of non-enzymatic model systems have been so disappointingly low that they inform poorly on the enzymatic reaction (Merenyi et al 1992).

Luciferases from all bioluminescent bacteria studied are heterodimers of α ($\approx$40 kDa) and β ($\approx$35 kDa) subunits. The *luxA* and *luxB* genes encoding the α- and β-subunits are adjacent in the *lux* operon, which also contains, among others, three genes (*luxC, D*, and *E*) encoding proteins that make up the fatty acid reductase complex (for aldehyde synthesis). *LuxA* and *B* have been cloned and expressed heterologously and extensively used as molecular reporters. There is a higher degree of similarity among α-subunits of different strains than between the β-subunits; the α and β of each strain are themselves quite similar (32% identity); β probably evolved by gene duplication. The organization of the complete *lux* regulon is of special interest (Baldwin & Ziegler 1992, Meighen 1994) and is discussed further below.

Figure 1 Bacterial and dinoflagellate bioluminescence. (*A*) In the bacterial reaction, the breakdown of the enzyme-bound peroxyhemiacetal E · FOOA is considered to involve electron transfer followed by charge annihilation resulting in the generation of the excited state (Eckstein et al 1993). (*B*) The structures of dinoflagellate luciferin and reaction products are known, but the reaction mechanism is not (Nakamura et al 1989).



The crystal structure of *Vibrio harveyi* luciferase has been determined at both 2.4 and 1.5 Å resolution, but only in the absence of substrates. A deep pocket on the α-subunit extending to the β-subunit may be the catalytic site (Fisher et al 1995, 1996).

Cnidaria (Coelenterates) and Ctenophores

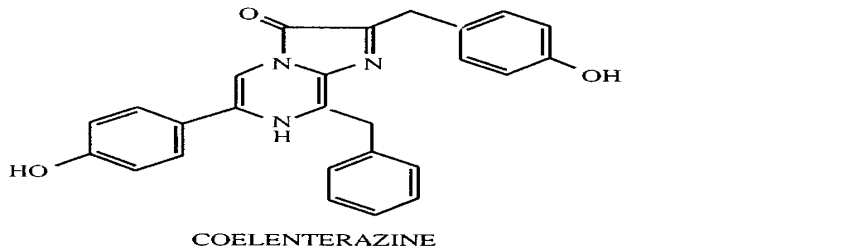
Many coelenterates and ctenophores, such as the sea pansy *Renilla*, the jellyfish *Aequorea*, the hydroid *Obelia*, and the ctenophore *Mnemiopsis* are bioluminescent. The emission is triggered by calcium, albeit by different mechanisms at the cellular and molecular levels. Within the coelenterates, recent findings indicate that the bioluminescent systems of the related hydrozoan and anthozoan classes may be quite different, even though they use the same luciferin, coelenterazine. Coelenterazine is an imidazolopyrazine, which occurs widely in luminous and non-luminous marine organisms (Shimomura et al 1980, Shimomura 1987, Thomson et al 1997). The luciferin of the crustacean *Vargula* is also an imidazolopyrazine, albeit with different substituents (Inouye et al 1992, Mager & Tu 1995).

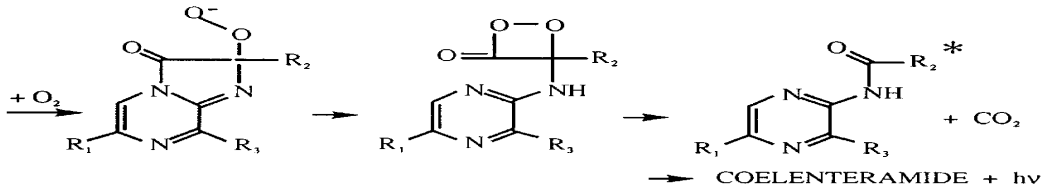
In *Renilla*, the anthozoan in which the biochemistry has been most studied, four proteins are involved (Cormier 1978). The first, a sulfokinase, removes a sulfate from the precursor (storage form?) of coelenterazine. The freed coelenterazine binds to a protein (luciferin-binding protein, 18.5 kDa, with three Ca^{2+} binding sites) and is released in the presence of calcium. Its oxidation is then catalyzed by *Renilla* luciferase (35 kDa); the reaction involves the formation of a dioxetanone intermediate, which breaks down to give CO_2 and oxidized luciferin (coelenteramide) in the excited state (Hart et al 1978) (Figure 2). The fourth protein is the green fluorescent protein, *Renilla* GFP, which (like the first two proteins) is not needed for the light-emitting reaction as such and, indeed, is not present in all anthozoans. In the absence of GFP, luciferase-bound excited coelenteramide emits blue light ($\lambda_{max} \approx 480$ nm). In its presence, the light emitted is green ($\lambda_{max} \approx 509$ nm).

When first isolated, the hydrozoan *Aequorea* system appeared not to have the classic luciferase/luciferin system, nor to require oxygen (Shimomura et al 1962). In the presence of a calcium chelator, one can isolate and purify a photoprotein, aequorin, which requires only calcium for light production. It is now clear that the photoprotein is simply a stable luciferase reaction intermediate to which an oxygenated form of the coelenterazine is already bound,

Figure 2 The bioluminescence reactions of both coelenterazine (*A*) and firefly luciferin (*B*) have been shown to involve the intermediacy of dioxetanone (Shimomura 1982). The hypothetical intramolecular electron transfer pathway is indicated in the firefly case (Koo et al 1978).

A





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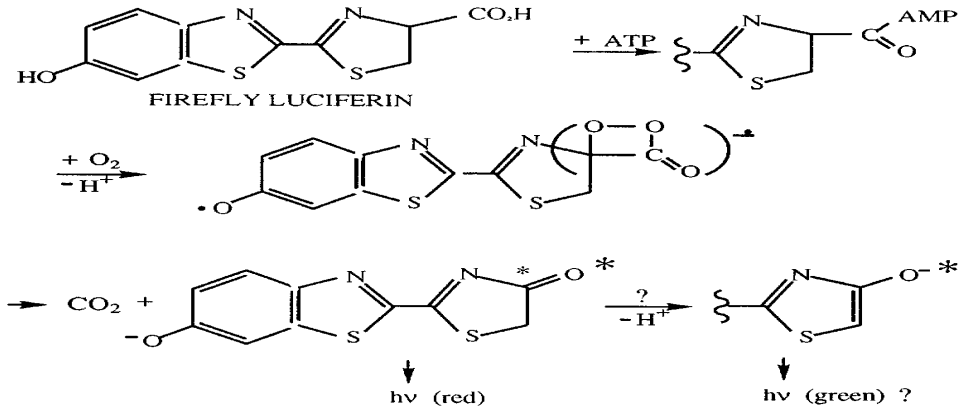


probably as a hydroperoxide. Calcium, for which the protein has three binding sites, triggers the flash by allowing the reaction to go to completion via the dioxetanone intermediate. Thus instead of triggering at the stage of luciferin availability, calcium acts here on a reaction intermediate.

As in the case of *Renilla*, the emission is blue (486 nm) when the *Aequorea* reaction is carried out in vitro, whereas the bioluminescence from the living organism is green (508 nm) because of the presence of the soluble green fluorescent protein, *Aequorea* GFP (Johnson et al 1962, Morin & Hastings 1971). This scenario is quite general: in coelenterates that emit in the green fluorescence band of GFP in vivo, the emission in extracts is in blue. Although the *Renilla* and *Aequorea* bioluminescence systems are similar, in fact they differ at the molecular level. Both utilize coelenterazine as luciferin (as do diverse organisms in distant phyla), and both make use of a GFP as a secondary emitter, but neither their luciferases nor their GFPs appear to be closely related evolutionarily (C Szent Gyorgyi, personal communication).

The three (12 amino acid long) Ca^{2+}-binding sites of aequorin, obelin (from the hydrozoan *Obelia*), and *Renilla* luciferin-binding protein are homologous to the Ca^{2+}-binding sites of other calcium-binding proteins such as calmodulin. Calmodulin has four such sites, where the spacing between sites 3 and 4 is the same as that between sites 2 and 3 of the three bioluminescence proteins. It is speculated that these genes had a common ancestor and that site 2 in calmodulin might have become the luciferin-binding site in the coelenterates' proteins (Tsuji et al 1995).

The involvement of energy transfer was first inferred in the case of *Obelia*, where, in extracts, photoprotein and GFP are found together in granules (Morin & Hastings 1971). If the cells are mechanically ruptured in sea water containing $MgCl_2$ (a Ca^{2+} antagonist), then centrifuged lightly to remove large cell debris, the supernatant contains both intact granules and the soluble "photoprotein" (i.e. the luciferase/luciferin hydroperoxide, Ca-triggerable system). If calcium is added to the supernatant, it causes a flash of blue light by reacting with the photoprotein. If water is now added, the granules osmotically rupture, and green light is produced as the photoprotein and its associated GFP come in contact with calcium. However, if the order of additions is reversed, first water, then calcium, only blue light is emitted, because the photoprotein-GFP complex has dissociated in dilute solution, thus preventing energy transfer.

Later experiments with *Renilla* showed that energy transfer still takes place quite efficiently in reaction mixtures containing as little as 0.1 μM GFP (Ward & Cormier 1976). At such a low concentration, energy transfer could take place only if the two proteins were pre-associated, because the average distance between non-associated proteins would be an order of magnitude too large to allow for radiationless transfer. Indeed, chromatography showed that

one luciferase molecule complexes with one GFP homodimer (Ward & Cormier 1978).

The study of coelenterate bioluminescence brought two remarkable benefits: GFP and aequorin. GFP is a uniquely valuable reporter of gene expression (see below), whereas aequorin provides a highly sensitive and rapid assay for intracellular calcium. The clone for apoaequorin has been expressed in different cells and can be visualized with added coelenterazine (e.g. Johnson et al 1995). *Renilla* luciferase is also used as a reporter (Lorenz et al 1991, 1996, Mayerhofer et al 1995).

Fireflies

Most bioluminescent insects are beetles (Coleoptera), in the families of Elateridae (such as click beetles), Phengodidae (the railroad worm with its red and green lanterns is a spectacular example) (Viviani & Bechara 1997), and Lampyridae, the fireflies. The reaction chemistry is presumably the same or similar for all beetles because their luciferases all react and give light with firefly luciferin (Wood 1995). This is not the case in bioluminescent Dipterae (such as the New Zealand glow worm), which are not discussed here.

Firefly luciferin is a benzothiazoyl-thiazole, an altogether different substrate from coelenterate luciferin, but again a dioxetanone is the critical energy-rich intermediate in the reaction. Luciferase first catalyzes the condensation of luciferin with ATP in the presence of Mg^{2+}, followed by the reaction of the adenylate with oxygen and cyclization of the peroxide; ATP provides the good leaving group AMP. The breakdown of the dioxetanone (rather than the hydrolysis of the adenylate) releases the energy, $\approx$50 kcal/mole, necessary to generate the excited state of oxyluciferin and CO_2 (Figure 2), with an overall efficiency reportedly close to 1 photon per oxidized luciferin (McCapra & Perring 1985, McElroy & DeLuca 1985). Even though the luciferin is the same in all beetles, their emissions span a wide-wavelength range, from green to orange (and even red in the railroad worm; note that the difference in energy between 560 and 630 nm photons is only $\approx$6 kcal/mol). Emission likely originates from the enzyme-bound mono-anion of oxyluciferin in its keto form, and the energy of its excited state, hence the color of the emission, probably depends on the tertiary stucture at the catalytic site (McCapra et al 1994; see below). The instability of free oxyluciferin in aqueous solution makes definitive assignments difficult.

Firefly luciferase is a 62 kDa monomeric protein with no prosthetic group. Its cDNA and that of several other beetle luciferases have been cloned and expressed in *Escherichia coli* and many eukaryotes (De Wet et al 1987, Masuda et al 1989, Wood et al 1989, Devine et al 1993). There is 40–50% sequence identity at the amino acid level between all luciferases of beetles belonging to the same family. Since the first step of the reaction is substrate activation

by formation of an adenylate, it is not surprising that firefly luciferase shows sequence similarity with other enzymes that also activate the carboxyl group of their substrates via adenylation. For example, 4-coumarate:CoA ligases and luciferase have 17% sequence identity, although they probably diverged more than a billion years ago (Schroder 1989, Wood 1995).

The crystal structure of firefly luciferase shows two distinct domains, a large N-terminal domain and a small C-terminal domain linked by a flexible, four-residue loop (Conti et al 1996). There is a cleft exposed to water between these two distinct domains. The residues that are most conserved among all beetle luciferases and the other ATP-activating enzymes are located on the surfaces facing this cleft and on the loop connecting the domains. The active site may be located in this region. However, the cleft is too wide to allow both surfaces to interact simultaneously with luciferin. On binding ATP or the adenylate, a conformation change probably occurs, water is excluded (the active site of luciferase is reported to be very hydrophobic; DeLuca 1969), and the substrate gets pinched in the cleft. Unfortunately, firefly luciferase is the only ATP-activating enzyme superfamily for which the crystal structure has been determined. Whether a further conformation change to utilize a new active site is required for the second function of luciferase, that of catalyzing the light-emitting reaction of the adenylated substrate with oxygen, is impossible to anticipate.

Dinoflagellates

The most studied of the many bioluminescent dinoflagellates, which are the unicellular algae responsible for much of the so-called phosphorescence of the sea, is *Gonyaulax polyedra*. Because its luciferin reacts with the luciferases of all dinoflagellates tested so far, it is likely to be representative of the group at large. The structure of dinoflagellate luciferin, determined from *Pyrocystis lunula*, shows no similarity to any other luciferin (Nakamura et al 1989). It is a linear tetrapyrrole probably derived from chlorophyll and very sensitive to autoxidation. The site of oxidation on the chromophore depends on whether the reaction is luciferase-catalyzed, and luminescence accompanies only the enzymatic reaction (Figure 1*B*).

The reaction product is not fluorescent, in contrast to unoxidized luciferin, which fluoresces brightly with a spectrum matching that of the bioluminescence ($\lambda_{max} \approx 470$ nm) (Hastings 1978). This paradox is not yet resolved. One possibility is that the bioluminescence is emitted by an excited transient intermediate, as in the bacterial reaction. Another is that an excited state formed in the reaction transfers its energy to still unreacted luciferin. However, studies indicate that only one luciferin molecule is required for light emission and the bioluminescence intensity in the in vitro reaction decays monoexponentially.

Two proteins are involved in *Gonyaulax* bioluminescence. One is a luciferin-binding protein (LBP), a dimer of two identical 75.5 kDa subunits, which sequesters luciferin at a physiological pH, protecting it from autoxidation, and releases it as the pH drops to 6 (Morse et al 1989b, Mittag et al 1997). The other, a luciferase (LCF) (137 kDa), is inactive at pH 8 and becomes active exactly in the pH range at which the LBP makes luciferin available for the reaction. The two proteins and luciferin are tightly packaged in special cytoplasmic organelles, the scintillons (Nicolas et al 1991, Desjardins & Morse 1993). The full-length cDNA of LBP and LCF have been cloned and sequenced (Lee et al 1993, Bae & Hastings 1994, Li & Hastings 1998). Both genes are present in many copies and neither has introns. They show no sequence homology with other proteins in the data bases, except for their N-terminal domains, which have a 50% sequence identity over a stretch of ≈100 residues. Interestingly, the luciferase contains three homologous and contiguous repeat sequences of 377 amino acids, and each of these sequences expresses a catalytically active peptide (Li et al 1997). In vivo, bioluminescence is emitted both as brief flashes (≈100 ms) and as a low intensity glow. The mechanisms involved in the control of emission at the biochemical, cellular, and circadian levels are discussed below.

WHAT DETERMINES THE EMISSION SPECTRUM?

The simplest scenario, which calls for the bioluminescence to be spectrally identical to the fluorescence of the reaction product (the oxidized substrate), fits the cases of coelenterates and ctenophores, such as *Mnemiopsis*, which do not have GFP, and the crustacean *Vargula*, which also uses an imidazolopyrazine as luciferin and oxidizes it via a dioxetanone. But more often than not this simple script is not followed, sometimes because of the presence of accessory proteins (see below). Another complication may arise if the immediate reaction product, is chemically unstable after emission. In that case, the emission corresponds to the fluorescence of an intermediate. As mentioned above, this occurs in bacterial bioluminescence and could well be taking place in dinoflagellate and firefly bioluminescence; nevertheless, in these cases it is still a chromophore directly formed in the excited state that emits the bioluminescence, even if it is not the final reaction product. The emission spectrum may also be affected by the protein environment, since the emitter is typically luciferase-bound.

The Enzyme: Firefly Luciferase

Mutagenesis of the luciferase cDNA of the Japanese firefly (*Luciola cruciata*) resulted in emissions ranging from the green to the red, as a consequence, in each case, of single amino acid substitutions (Kajiyama & Nakano 1991). For

example, the substitution H433Y shifted the peak of emission from 562 nm to 612 nm, while the emission bandwidth remained unchanged. Four of the five residues that singly caused color shifts upon substitution are located along the second half of the sequence in the large N-terminal domain, and the fifth is in the C-terminal domain. In the case of click beetle luciferase isozymes, expression in *E. coli* of four different cDNAs coding for proteins differing in only a few amino acids resulted in peak emissions ranging from green to orange; these substitutions were all in a short region of the the N-terminal domain (Wood et al 1989).

To understand how the substitution of a single residue can radically affect the emission spectrum would require knowing, besides the exact location of these residues vis-à-vis the active site, the chemical identity of the excited reaction product. It is assumed that the excited mono-anion of the keto form of oxyluciferin emits red light (Wood 1995). It had been thought that the wild-type yellow emission resulted from enolization during the lifetime of the excited state, at physiological pH 8. Indeed, the 5,5′-dimethyl analog of luciferin, which cannot enolize, emits only red light. It was also thought that low pH ($\approx$6) results in red light because of more extended conjugation. Oxyluciferin is so extremely unstable that it is practically impossible to confirm experimentally these conclusions (White & Roswell 1991).

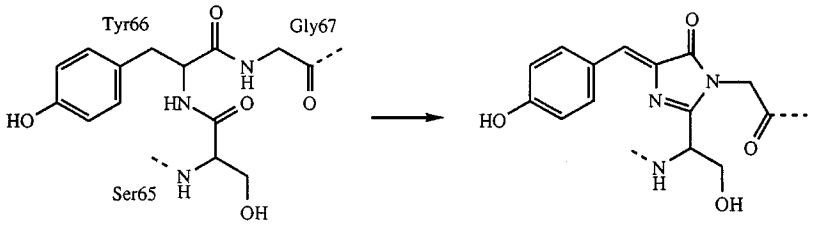
The previous work had thus focused on the identity of two discrete emitters, one emitting yellow light, the other red. But the observation that naturally occurring and mutant luciferases emitted in a whole range of colors, each with an emission spectrum consisting of a narrow, shoulderless band, cannot be accounted for by the superposition of the spectra of these two emitters. In fact, the effect of protein environment on the color of emission may be due to restriction on the conformation of luciferin (McCapra et al 1994, McCapra 1997). While the molecule is probably quite insensitive in the ground state to the angle of twist between the two rings around the C2-C2′ axis, in the excited state the energy minimum is estimated to correspond to a structure with the rings at a 90° angle. When a twisted excited oxyluciferin anion emits, it ends up in a twisted ground state because conformation changes are too slow to compete with emission of a photon; the energy of the electronic transition will then correspond to photons toward the red end of the spectrum. But if the luciferin intermediate is constrained by luciferase to be nearly planar, the energy of the emitted photon will be larger, i.e. blue-shifted. Thus the degree of twist might determine a continuum of emission colors.

Accessory Proteins: GFP

In some systems, the bioluminescence spectrum may not match the fluorescence of the luciferase reaction product because of the presence in vivo of an

accessory fluorophore. The most famous of such "antenna" proteins is coelenterate GFP.

The GFP proteins turn out to be remarkably interesting. Their most unusual (and valuable) feature is that the chromophore is covalently bound. In *Aequorea* GFP, it results from the post-translational cyclization, dehydration, and oxidation of residues Ser65-Tyr66-Gly67 in the 238 amino acids protein (Heim et al 1994). The cloning of a cDNA of this GFP (Prasher et al 1992), and the demonstration that its expression in prokaryotic or eukaryotic cells produces a fluorescent protein, thus without the need for any coelenterate-specific enzymes, opened the gates to innumerable applications of GFP as a reporter gene and a marker of cellular localization (Chalfie et al 1994).



GFP chromophore

Even though the GFP peptides of *Aequorea* and *Renilla* are not strongly homologous (Cubitt et al 1995; C Szent Gyorgyi, personal communication), their imidazolinone chromophores are identical, and the fluorescence spectra of both consist of a narrow band at 509 nm. However, the absorption spectrum of wild-type *Aequorea* GFP shows two bands, a major peak at 395 nm and a minor one at 475 nm, whereas that of *Renilla* GFP consists of a single peak at 498 nm, fivefold more intense than the 395 nm peak of *Aequorea* GFP. This in itself demonstrates, as for the visual pigments, how much the protein environment of a chromophore may influence its spectral properties. The numerous mutants of *Aequorea* GFP have now made this abundantly clear, while making available custom-made GFP of specially desirable spectroscopic properties (Heim & Tsien 1996). Mutations in GFP far removed from the chromophore can affect the absorption and fluorescence spectra, but the near-full-length gene is essential: Only one amino acid can be deleted at the N terminus and at most 15 at the C terminus without loss of fluorescence (Cubitt et al 1995).

Within the chromophore itself, mutations of either Ser65 or Tyr66 yield fluorescent proteins displaying only one absorption band. In the case of Ser65 mutants, the short-wavelength band is lost with little spectral shift of either the 475 nm band or the emission maximum; the properties of this particularly useful S65T mutant are very close to those of *Renilla* GFP. In mutants of Tyr66,

the 475 nm band is lost, while the fluorescence is shifted to the blue by as much as 60 nm in T66H. In contrast, Gly67 cannot be substituted without complete loss of fluorescence.

The biosynthesis of the chromophore can be looked upon as requiring successive steps: proper folding of the peptide chain so as to bring residues 65 and 67 to the geometry appropriate for cyclization, dehydration, and finally oxidation to form a C=C double bond on the phenolic side chain of tyrosine and thus create the 4-hydroxycinnamyl part of the chromophore. If GFP is expressed in anaerobically grown *E. coli*, the correct molecular weight protein is produced, but it is not fluorescent. The subsequent appearance of fluorescence requires only the admission of oxygen and will occur in extracts; it develops relatively slowly, in a matter of hours (Heim et al 1994). The mechanism of this slow oxidation step is unknown, other than that it requires no enzyme since it occurs even in very dilute lysates. It deserves particular mention because it limits the use of GFP as a fast reporter of gene expression.

The kinetics of chromophore formation in mutant S65T expressed in *E. coli* was studied by comparing soluble mature GFP, where the chromophore is already formed, with the nonfluorescent GFP extracted from inclusion bodies, where improper folding prevents cyclization of the chromophore (Reid & Flynn 1997). Neither of these proteins is fluorescent after denaturation. Upon renaturation, the soluble protein reacquires its fluorescence in a matter of minutes since its chromophore is preformed. In contrast, the GFP extracted from inclusion bodies requires hours, because after protein folding and cyclization of Thr65 and Gly67, the slow oxidation step must still take place. The rate of protein folding seems to be the same in these two GFPs, as shown by the same rate of acquisition of resistance to proteolysis following denaturation/renaturation.

Many of the properties of GFP, such as its thermal stability and remarkable resistance to proteolysis, derive from its unique structure. The crystal structures of WT GFP (Yang et al 1996, Brejc et al 1997) and mutants S65T (Ormö et al 1996) and Y65H/Y145F (Wachter et al 1997) show the chromophores located at the center of a protein cylinder, dubbed a β-can. The S65T crystals are monomeric, whereas WT GFP forms monomeric or dimeric crystals made of two parallel cans (30 $\times$ 40 Å), each with its central chromophore protected by 11 β-strands cylindrically wrapped around it. Solvent access to the inside cavity in these "lanterns" is blocked on top and bottom by short segments of α-helices, although some water molecules are immobilized inside; there is clearly no room for an enzyme to catalyse chromophore formation. The GFP dimers seen in the crystal result from hydrophilic interactions between three of the β-strands resulting in many hydrogen bonds. There is also a small hydrophobic patch that may play a role in the interaction of GFP and aequorin. The state of association of aequorin and GFP in vivo is unclear. In solution, the association

depends on both concentration and ionic strength. It is reported that GFP and aequorin may form a heterotetramer (Cutler & Ward 1997).

Surprisingly, the aequorin-GFP system seems less than ideal for energy transfer. Of the two absorption bands of WT GFP, at 395 and 475 nm, only the weaker long-wavelength band overlaps significantly with the emission band of aequorin. The situation is more favorable in *Renilla*, and it has inspired the constructions of pairs of *Aequorea* GFP mutants with spectral properties specifically suited to their use as donor-acceptor tags in studies of protein-protein interactions in vivo. In two ingenious fluorescence experiments mimicking in vivo aggregation, donor and acceptor pairs, each made of a blue- and a green-shifted GFP mutant, were linked together by flexible chains of 20 or 25 amino acids, which included a protease-sensitive site. After addition of the specific protease, the fluorescence gradually shifted from green to blue as the two GFPs separated and resonance energy transfer became less efficient (Heim & Tsien 1996, Mitra et al 1996).

The photophysics of *Aequorea* GFP are complex and interesting. The intensity ratio of its two absorption bands depends on pH, ionic strength, and temperature. They originate from the ground states of two different forms of the chromophore, which can photoconvert (during the course of hours under laser irradiation). Excitation in the high-energy band (<400 nm) results in its bleaching, with a concomitant increase of absorbance in the lower energy band and an isosbestic point at 425 nm. This process is only partially and slowly reversible (days) in the dark. The two forms differ by their state of ionization associated with the network of hydrogen bonds tying the chromophore to the rest of the protein. The presence of the threonine methyl in S65T is sufficient to keep the chromophore permanently in its ionized form and prevent photoisomerization (Chattoraj et al 1996, Brejc et al 1997).

Accessory Proteins: YFP and BFP

It is not known whether the association of aequorin and GFP, which modifies the color of the emission, also alters the kinetics of the reaction, indicative of a chemical interaction prior to excitation. Such interactions do occur in the case of two accessory proteins in bioluminescent bacteria, where in contrast to GFP, the chromophores are not covalently bound. While in vitro emission from the bacterial luciferase-catalyzed reaction peaks at ≈495 nm, the in vivo emission is blue shifted to ≈475 nm in *Photobacterium phosphoreum* and *P. leiognathi*. This is due to the presence of a protein (LumP, 21 kDa) in which the fluorophore is 6,7-dimethyl-8-(1′-D-ribityl)lumazine, a precursor of riboflavin (Koka & Lee 1979). A yellow fluorescence protein (YFP, 28 kDa), with FMN as chromophore, causes a strain (Y-1) of *Vibrio fischeri* to emit yellow light ($\lambda_{max} \approx 540$ nm) (Daubner et al 1987, Macheroux et al 1987).

In vitro, these two functionally analogous proteins shift the emission peaks of the corresponding luciferase reactions in a concentration-dependent manner. LumP and YFP are homologous (O'Kane et al 1991) and also share homology with riboflavin synthase, which binds two molecules of lumazine (O'Kane & Prasher 1992). YFP and LumP do not simply acquire their excitation energy by energy transfer from the luciferase-hydroxyflavin, the normal emitter, but influence the enzymatic reaction at an earlier stage. YFP, for example, accelerates up to 10-fold the rate of intensity of decay of the in vitro reaction of *V. fischeri* luciferase, in a concentration-dependent manner (Eckstein et al 1990). LumP has a similar but smaller effect on the reaction of *P. phosphoreum* and *P. leiognathi* luciferases (Petushkov et al 1996a,b). Neither the apoproteins nor the chromophores alone are effective, nor do YFP or LumP associate with luciferase itself. Therefore, the fluorescence proteins must interact with and destabilize an enzyme-bound intermediate, such as the peroxyhemiacetal, and deviate the reaction course.

In the case of LumP, the luciferase-bound emitter it thought to be generated in its excited state while complexed with LumP, the excited state of which is created by energy transfer, resulting in blue emission. However, the effects of LumP and YFP on the rate of intensity decay, described above, are not entirely consistent with this mechanism. For YFP there is a further observation. If there were a single primary emitter of nanoseconds lifetime and it either emitted blue light or transferred its energy to YFP, the time courses of blue and yellow emissions should be identical on the experimental time scale of seconds (in the single-turnover standard assay). This, however, is not observed: In the presence of YFP, the onset kinetics of blue emission (from the luciferase-bound hydroxyflavin emitter) and yellow emission (from YFP) are clearly different. Thus a single kinetic pathway and primary emitter with an ultimate energy transfer process to YFP can definitely not account for the results (Eckstein et al 1990). The blue emission must originate from that fraction of peroxyhemiacetal that has not reacted with YFP, whereas yellow emission originates from the complex, via an intra-complex energy transfer process.

The effect of aldehyde chain length on the reaction rate, with or without YFP, supports the assumption that the critical intermediate with which YFP (and presumably LumP) interacts is the peroxyhemiacetal. If a fast rate of intensity decay, as with dodecanal and tetradecanal, reflects a short lifetime for this intermediate, then the chances of its interception by YFP will be the poorest. Indeed, at a given concentration, YFP is relatively more effective with aldehydes of shorter chain length (Sirokmán & Hastings 1997).

The effect of YFP is prominent at 4°C and virtually absent at 20°C. One possibility is that a dimeric inactive form of YFP is favored by higher temperature. Another is that a change in YFP conformation could affect its ability to attain a critical geometry at the active site (Sirokmán et al 1995).

Finally, the case of YFP illustrates, once again, how much a protein can alter the emission spectrum of its noncovalently bound chromophore, in this case FMN. The λ_{max} of free FMN fluorescence is 525 nm, whereas in YFP it is red-shifted to 540 nm, possibly by the conformation changes in the isoalloxazine moiety imposed by protein binding (Karatani & Hastings 1993).

CELL BIOLOGY AND CELLULAR CONTROL OF LUMINESCENCE

As the biochemistry of different luminescent systems differs, so do the structural and regulatory aspects at the cellular level (Hastings & Morin 1991). In some cases, the luciferase system appears to be distributed throughout the cytoplasm, whereas in others the cells (photocytes) specialized for bioluminescence possess organelles responsible for light emission. Still other systems remain uninvestigated.

Bacteria

Bacteria emit light continuously, and their small size would seem to argue against any subcellular localization of the light-emitting system; this has been confirmed by immunogold labeling (Colepicolo et al 1989b). Reports that bacteria emit light in pulses (Berzhanskaya et al 1975) were intriguing because bacterial luciferase constitutes a shunt of the electron transport pathway, which could involve some kind of feedback regulation with cyclic bursts. However, careful measurements of emission from a single bacterium established that the light emission is indeed continuous (Haas 1980).

Cnidaria

Cnidarian photocytes may be clustered or widely scattered, most often located in or near the endoderm. In *Obelia*, action potentials in the conducting epithelium result in the release of calcium in the photocytes, which triggers flashing. An inward calcium current occurs in depolarized support cells, not the photocytes, and gap junctions between the two provide channels for calcium entry to the photocytes (Dunlap et al 1987). In extracts of hydrozoan and anthozoan, luminescence activity occurs in both particulate and soluble fractions (see above; Morin & Hastings 1971), suggesting that an organelle is involved. Although some evidence supported this possibility (Anderson & Cormier 1973, Spurlock & Cormier 1975), further studies did not (Case & Strause 1978). The particles might represent cytoplasmic vesicles formed from endoplasmic reticulum during extraction.

Fireflies

Adult firefly lanterns are comprised of stacked units in which the photocytes are arranged in rosettes, with a central cylinder through which run branches of

the lantern nerve and tracheae that carry oxygen to the cells (Case & Strause 1978). The lantern itself comprises a series of such rosettes, stacked side-by-side in many dorso-ventral columns. Based on immunochemical labeling, organelles containing luciferase in the photocytes of the American firefly were identified as peroxisomes (Hanna et al 1976). Indeed, the C-terminal tripeptide SKL peroxisomal targeting sequence is present in luciferase (Gould et al 1989), and when the gene is expressed in yeast or mammalian cells, luciferase localizes to peroxisomes (Keller et al 1987, Aflalo 1990, Soto et al 1993). The SKL sequence is absent in the Japanese firefly luciferase (Masuda et al 1989).

Although flashing is initiated by a nerve impulse, the nerve terminals in the light organ are not on photocytes but on tracheolar cells, which may regulate the supply of oxygen, suggesting that this controls the flash (Ghiradella 1977). However, the rapid kinetics and bunched flashes, complex wave forms, and high-frequency flickering all seem unlikely to be regulated by access to oxygen. Equally baffling is how a membrane-bound cytoplasmic organelle could be linked to an excitatory process, be it transmitted via a membrane or a diffusible reagent. A novel discovery may be in store.

Dinoflagellates

Bioluminescence in dinoflagellates is emitted from many (≈400 per cell) small (≈0.4 μm) organelles, the scintillons. It has been studied extensively in only one species, *G. polyedra*; the ultrastructure and flash control mechanism are fascinating and novel. Scintillon luminescence, visualized by image intensification, colocalizes with the fluorescence of luciferin (Johnson et al 1985). Identified by immunolabeling (Nicolas et al 1987a), scintillons are spherical vesicles projecting into the cell vacuole. This preserves the continuity of the vacuolar membrane conducting the triggering action potential (Eckert 1965), which is postulated to open proton channels. This causes a transient pH change in the scintillons, the activation of the reaction, and a flash (Hastings & Dunlap 1986).

The two major proteins, luciferase (LCF) and luciferin-binding protein (LBP), are localized to the scintillons (Nicolas et al 1991). Upon cell extraction at pH 8, activity can be obtained from both soluble and particulate fractions. The latter is attributed to scintillons that seal off at the neck to form closed vesicles, which can be purified by density gradient centrifugation. Upon a shift to pH 6, they emit flashes closely mimicking the in vivo flash, with kinetics independent of concentration. These vesicles can be recharged in vitro by incubating with luciferin. The soluble fraction contains luciferase and the luciferin-LBP complex, but emits light only upon a shift to pH 6, which activates the luciferase-catalyzed oxidation of the released luciferin; in this case, the kinetics of the luminescence

depends upon luciferase concentration (Hastings 1978). The components of all luminous dinoflagellates studied cross-react biochemically, and all have scintillons, as judged by in vitro assays in extracts (Schmitter et al 1976) and immunocytochemical labeling (Nicolas et al 1987b). One significant difference between species is the the absence of lucifern-binding protein in *Pyrocystis*, yet flashing is still tightly controlled (Colepicolo et al 1993).

Luminescence in *G. polyedra* and other dinoflagellates is regulated by an endogenous circadian clock, and is maximum during the dark (night) phase (Johnson & Hastings 1986). Remarkably, both luciferase and luciferin-binding protein in *G. polyedra* are destroyed at the end of the night phase and then synthesized again in the next cycle. Moreover, the scintillons themselves are broken down and reformed each day (Fritz et al 1990); the circadian cycle may actually be viewed as a daily differentiation of certain cellular processes.

The synthesis of the two luminescence proteins LCF and LBP of *Gonyaulax* is regulated translationally; their mRNA levels and translatabilities remain constant over the circadian cycle (Morse et al 1989a, Mittag et al 1998). A protein of ≈45 kDa has been demonstrated to bind to the 3′UTR of the *lbp* mRNA at an unusual 22 nt sequence containing seven U(U)G repeats (Mittag et al 1994) and is hypothesized to regulate 5′ initiation (Mittag et al 1997). The circadian mechanism and how it regulates cellular processes remains one of the real enigmas in biology. Synthesis and destruction is not the only mode of regulation, even in dinoflagellates. For example, in *Pyrocystis*, the amount of luciferase remains constant over the cycle (Knaust et al 1998), but its cellular location and responsiveness change from night to day (Widder & Case 1982).

Polynoid Scale Worms

Scale worms are marine annelids possessing two rows of scales covering the dorsal surface of the animal. In luminous species the cells of the ventral epithelial layer of the scales are photocytes. When attacked by a predator, the scales first emit rapid flashes so as to frighten and deter, but they may then be shed in the water while the animals themselves escape, leaving the now glowing scales as luminous decoys. Little is known of the biochemistry. A fluorescence attributed to flavin develops in proportion to the amount of light emitted (Bassot & Bilbaut 1977) and a photoprotein (≈500 kDa), which emits light upon the addition of superoxide radicals, can be extracted from the cells (Nicolas et al 1982, Colepicolo et al 1990).

Within each photocyte are some 30 to 50 luminous organelles (1 to 5 μm in diameter) called photosomes, arranged in concentric rows around the central nucleus (Pavans de Ceccatty et al 1977). The photosomes are made of tubules of endoplasmic reticulum, 20 nm in diameter, structured as regular paracrystalline arrays. Flashes (≈100 ms) are triggered by calcium entry

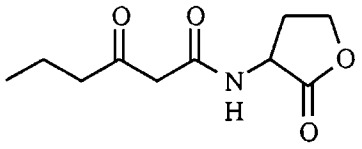
accompanying epithelial action potentials; conduction is facilitated by numerous gap junctions. A striking feature is that in isolated scales, repetitive stimuli (e.g. 1 Hz) result in progressive increases in flash intensity, attributed to the recruitment of more and more photosomes. This results from the rapid (ms) formation of new dyad junctions, thereby coupling additional photosomes to the excitatory plasma membrane (Bilbaut & Bassot 1977, Bassot 1987). The recruitment may be observed photometrically by luminescence and fluorescence of individual photosomes. After many (20–30) stimuli, fatigue occurs and flash intensity decreases. A single strong stimulus can evoke a similar train of flashes with a similar pattern of increasing then declining intensities. Paracrystalline arrays structurally similar to photosomes are also found in photoreceptors (Eakin & Brandenburger 1975, Bassot & Nicolas 1978).

REGULATION OF BACTERIAL BIOLUMINESCENCE

Autoinduction, Intercellular Chemical Signaling, and Quorum Sensing by Bacteria

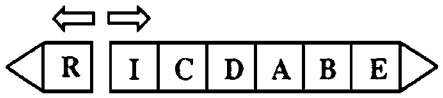
We owe to bioluminescent bacteria the discovery of a clever way of intercellular communication. Why would isolated bacteria, free-living at sea, spend energy to emit light? The answer is, they do not, because the relevant genes are never turned on. We now know that luminescent, as well as a variety of non-luminescent, bacteria start the specific transcription of some genes only when the cell density is high enough for the products of these genes to have an impact on the environment. In culture, *V. harveyi* and *V. fischeri* first grow without synthesizing luciferase (and therefore without luminescing) until mid or late exponential phase is attained and a cell density threshold is crossed, after which luciferase is expressed and the cells luminesce. A not-yet luminescing, low density culture can indeed be activated to synthesize luciferase and luminesce by the addition of medium from a late culture (Nealson et al 1970). This medium contains a freely diffusible pheromone, the autoinducer, which is synthesized by the bacteria and accumulates in the medium as the cells grow. Thus by sensing the level of autoinducer, the cells are able to estimate their density and to initiate the energetically costly synthesis of luciferase (and the whole bioluminescence system) only when they are numerous enough to be seen, as when cultured in the light organ of a host such as a fish or a squid (Boettcher & Ruby 1995).

In *V. fischeri*, the autoinducer (VAI) is an N-acyl-homoserine lactone (Eberhard et al 1981).



VAI

Its regulon, which has been cloned and expressed in *E. coli*, contains two regulatory genes, *luxI* and *luxR*, in two divergent operons (Meighen 1994, Ulitzur & Dunlap 1995, Fuqua et al 1996). The product of *luxI* on the rightward operon (which contains *luxA* and *luxB* for the two luciferase subunits, as well as *luxC*, *D*, and *E* for aldedyde synthesis) synthesizes the autoinducer from S-adenosylmethionine and an acyl-acyl carrier protein (Moré et al 1996, Hanzelka & Greenberg 1996, Schaefer et al 1996). The *luxR* gene on the leftward operon codes for the LuxR protein, whose N-terminal domain binds the autoinducer (Hanzelka & Greenberg 1995); this causes the LuxR C-terminal domain to act as a transcriptional regulator, by binding DNA in synergy with RNA polymerase at a palindromic sequence located upstream of the transcription start of the rightward *lux* operon (Stevens & Greenberg 1997).

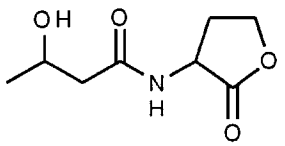


When the concentration of VAI in the medium (and therefore within the cell) is below a threshold, transcription of the rightward operon goes on at a steady but low level, allowing for the build up of autoinducer concentration to the point where VAI associates with *luxR* and activates transcription of both operons in an autocatalytic feedback loop (Eberhard et al 1991, Dunlap 1992). Some apparent contradictions remain, possibly because most studies have been done with the *lux* regulon cloned in *E. coli*, which may be lacking some elements important for the specificity of the autoinducer (Sitnikov et al 1995).

A growing list of other gram-negative, non-bioluminescent bacteria are now known to utilize a signaling system based on various homoserine lactones and homologs of LuxI- LuxR-type proteins, although often more complex than the autoinduction scheme of *V. fischeri* outlined above (Swift et al 1996). Among these are *Pseudomonas aeruginosa*, in which two systems, LasI-LasR and RhlI-RhlR, control the generation of several virulence factors, notably in the lungs of cystic fibrosis patients; *Agrobacterium tumefaciens*, a plant pathogen,

in which TraI-TraR regulates the conjugate transfer of oncogenic plasmids into cell nuclei; and *Erwinia carotovora*, utilizing ExpR-ExpI to activate the synthesis of antibiotics and of plant cell-wall degrading exoenzymes in plants. Remarkably, in all these bacteria the signaling pheromones are also N-acyl homoserine lactones, differing from the *V. fischeri* autoinducer only by the structure of the acyl groups. It is therefore not surprising, for example, that there is a significant degree of homology between LuxI, LasI, TraI, and ExpI. The R proteins are also homologous but to a lesser degree. Although the purple bacterium *Rhodobacter sphaeroides* is not known to associate with a eukaryotic host, a homoserine lactone autoinducer has recently been shown to prevent aggregation (Puskas et al 1997), adding to the remarkable generality of this mode of transcriptional regulation.

This summary grossly oversimplifies the complexities of quorum sensing as now understood. For instance, there is evidence that *V. fischeri* responds to more than one autoinducer, and this is certainly the case in the other most studied bioluminescent *Vibrio* species, *V. harveyi*, in which autoinduction was first discovered (Nealson et al 1970). One of its autoinducers, HAI, is a N-acyl-homoserine lactone (Moré et al 1996), a close analog of VAI, yet the genes in *V. harveyi* serving the functions of *luxR* and *luxI* are not a part of the *lux* operon,



HAI

are not homologous to *luxR* and *luxI*, and VAI and HAI do not cross react (Bassler et al 1993). In contrast, the same N-acyl-homoserine lactone may be utilized by a number of different species. For example, VAI is a signaling molecule not only in *V. fischeri*, but also in *E. carotovora* and several other bacteria. This apparent lack of specificity may relate to interactions between species. Bacteria may, for example, have the ability and an advantage in monitoring the population of other species, as well as in sensing their own density (Fuqua et al 1996).

Among non-luminous bacteria utilizing a homoserine lactone as autoinducer, the insect pathogen *Xenorhabdus nematophilus* is of special interest (Dunphy et al 1997). These bacteria live in the gut tract of a parasitic nematode, which invades the hemocoel of insect larvae, where the bacteria are released and then grow, killing the insect. It was recently shown, by comparison of the virulent and an avirulent mutant of *X. nematophilus*, that virulence is regulated by HAI,

the *V. harveyi* autoinducer; the avirulent mutant becomes virulent if injected in an insect together with HAI. In contrast, the regulation of luminescence of a very similar bacterial symbiont of nematodes (also an insect pathogen), *Photorhabdus* (formerly *Xenorhabdus*) *luminescens*, exhibits an autoinduction-like pattern but appears not to involve an autoinducer; it is speculated that regulation occurs post-transcriptionally (Hosseini & Nealson 1995). Another strain of *P. luminescens* has been isolated from human wounds; it also appears to autoinduce its luminescence, but nothing is known of the mechanism involved (Colepicolo et al 1989a).

What Can Luminous Bacteria Teach Us About Symbiosis?

The complexities of the association of *P. luminescens* and its nematode host were recently reviewed (Forst & Nealson 1996). They bring up the basic questions posed by symbiosis in the case of bioluminescent bacterial symbionts and their hosts. Several of these are addressed in a beautiful system particularly well suited to studies at the molecular, cellular, and morphological levels, the sepiolid squid *Eupryma scolopes*.

Its light organ hosts contains a dense culture ($>10^{10}$ cells/ml) of a specific strain of *V. fischeri* bacteria (Ruby & McFall-Ngai 1992, Ruby 1996). Both host and symbiont can be cultured in the laboratory, and the mutual benefits they derive from the association are easily understood: The squid is provided with a behaviorally useful light source, and the bacteria are given room and board and disseminated. The light organ of newly hatched offspring is bacteria-free and must therefore acquire symbionts from the surrounding seawater, in the only case where this mode of initiation of symbiosis has been unambiguously established.

In a young, still symbiont-free squid exposed to seawater containing *V. fischeri* cells, a special ciliated microvillous epithelium moves the sea water past pores leading to the empty crypts of the organ, presumably to facilitate entry of the bacteria. This induces development programs in both the organ and the symbionts; exposure to a very few bacteria for only a few hours is enough to start this colonization process. The crypts grow, the cells of the ciliated microvillous structure undergo alterations, then regress, and after 4 days die by bacteria-induced apoptosis (McFall-Ngai & Ruby 1991, Montgomery & McFall-Ngai 1994). None of these developments occur if the new organ is not exposed to competent bacteria. As for the bacteria, they immediately start proliferating rapidly, but after 12–24 h their growth rate declines, their size decreases, and they lose their flagella (Ruby & Asato 1993). Every morning the squid expels up to 90% of the bacteria from its organ (Boettcher et al 1996), which is repopulated by bacterial growth, while the expelled bacteria are viable and regrow flagella.

Of the thousands of bacteria from which to choose, many of which are luminous, how is the specific species and strain selected? There is as yet no answer to this fundamental question, but one speculation is that luminous bacteria are selected because the luciferase system detoxifies harmful reactive oxygen species (ROS), which may be generated in abundance by the host's light organ. Indeed, tissues lining the organ are rich (≈250-fold richer than other squid tissues) in a peroxidase homologous to human myeloperoxidase (Weis et al 1996), which catalyzes the reaction of H_2O_2 and Cl^- to produce the more potent bactericidal agents HOCl and singlet oxygen (Steinbeck et al 1992). A role for luciferase is indicated by the fact that while mutants lacking *luxA* are capable of colonizing the light organ, their population soon drops to 10% of normal. Mutants defective in either *luxI* or *luxR* behave in a similar way. They colonize the light organ normally, although with a significantly reduced light output, but 48 h later their population is down to 10%. However, this does not explain how the selection of a particular luminescent strain is achieved. Another possibility is that the ROS induce the specific transcription of resistance genes (Khan & Wilson 1995) present only in the competent symbiont strain, meaning that the host peroxidase is part of a signaling system important in the establishment of the symbiosis.

Different strains of a given luminous species may differ in competence. As noted earlier, the *E. scolopes* organ can be colonized only by *V. fischeri*, including strains isolated as symbionts from fishes or other squids. Interestingly, if newly hatched squid are exposed separately to either of two strains of *V. fischeri* found in the Hawaiian coastal waters, where the squid lives, both are able to successfully colonize the light organ. However, if they are exposed to a mixture of the two, the *V. fischeri* strain normally found in the squid's organ—and paradoxically the far less luminescent of the two in laboratory cultures—soon outcompetes the other and becomes completely dominant, leading to a monoculture (Lee & Ruby 1994).

Recent work illustrates the importance of studies of the *V. fischeri-E. scolopes* symbiosis and their probable significance in the general field of pathogenicity. *V. fischeri* was found to have a gene homologous to the *Vibrio cholerae* transcription activator *toxR*, which regulates the production of the cholera toxin. *V. fischeri* also secretes an enzyme, halovibrin, which, like cholera toxin, has ADP-ribosyltransferase activity. However, halovibrin and cholera toxin share no sequence similarity (Reich & Schoolnik 1996). This is an intriguing finding, especially with regard to the pathogenic role of enzymes with ADP-ribosyltransferase activity.

Lastly, the case of the *V. fischeri-E. scolopes* association answers a general question in symbiosis: How is the symbiont population maintained within acceptable limits, so as not to overgrow the host? The daily dumping of 90% of the bacteria from the light organ appears to be the answer here.

In contrast to *V. fischeri*, *P. leiognathi*, and *P. phosphoreum*, which also colonize the light organs of fishes and squids, *V. harveyi* has never been found as a light organ symbiont. As a marine enterobacterium, it occurs in the gut of fishes and invertebrates. By attracting organisms to ingest fecal pellets that would otherwise sediment to the bottom, its luminescence may serve to aid in the dispersion and propagation of the bacteria, a rather unglamorous return for the cost of emitting light.

PERSPECTIVES

Evolutionary aspects of bioluminescence remain among the most intriguing of the unknowns. Based on the number of different extant systems, it has been estimated that light emission originated independently at least 30 times, from bacteria, fungi, and algae all the way to squid and fish (Hastings 1983). Furthermore, it is reasonable to assume that even more bioluminescence systems might have evolved but did not survive, for light emission is not essential for life. While in some cases, such as fungi, the function of bioluminescence is not obvious, nor is its mechanism (Shimomura et al 1993), in others it is clear that light is meant to be seen and used for various different functions, such as courtship or to repel predators or attract prey.

That bioluminescence is not found in higher vertebrates or plants, where it could also be functionally important, suggests that some barrier to the de novo creation of bioluminescence came into play. This might be regarded as in keeping with the great preponderance of luminescence in marine organisms. Bioluminescence is indeed abundantly present among fishes: Some have light organs harboring luminous bacteria, others have photophores utilizing coelenterazine or *Vargula* luciferin (which they may acquire nutritionally).

Through what circumstances did luminous bacteria become uniquely successful as symbionts in higher organisms? Since non-bioluminescent dinoflagellates engage in symbiotic associations with higher organisms such as clams and corals (which harness them for photosynthesis), why have bioluminescent species not been co-opted to provide light? What are the traits critical to a symbiont-host relationship? Some of these questions are being asked in the case of the squid.

An old idea, that bioluminescence did not first evolve for the production of light, but as a mechanism of detoxifying an atmosphere becoming dangerously aerobic (McElroy & Seliger 1962), keeps resurfacing in various forms (Seliger 1975, Seliger 1987, Barros & Bechara 1998). The finding that coelenterazine, widespread in luminous and non-luminous marine organisms, is a potent antioxidant suggests that protection against reactive oxygen species could have been its primary role (Rees et al 1998). Enzymes that would rapidly channel the energy liberated by the oxidation of coelenterazine into harmless photons might

then have been selected. In another example, bacterial luciferase expressed in *E. coli* acts as a source of superoxide in the absence of the bioluminescence substrate decanal, not in its presence (Gonzalez-Flecha & Demple 1994).

The chemistry of all known bioluminescence reactions remains punctuated with question marks. There is no definitive picture yet of the elementary steps that culminate in the release of the large reaction energy (≈50 kcal) as light. This is not surprising, considering that the mechanism of only a few of the simplest reactions is understood at the molecular level. In chemiluminescence and bioluminescence, the involvement of electron and charge transfer processes remains an area of discussion, with no definitive answers. Progress will require the development of more responsive model systems and more incisive techniques. Because chemi- and bioluminescence are, by definition, triggered chemically rather than by light, they are not amenable to the panoply of ultrafast laser-based photophysical methods now used to probe photochemical processes.

One can anticipate from the diversity of luciferins and luciferases that common characteristics will emerge that will help elucidate the critical features of chemiexcitation. In any case, the mechanistic uncertainties do not detract from the uniqueness of bioluminescence as a non-invasive and immediately responsive probe of a reaction or molecular marker. The extreme sensitivity of light-detecting systems brings analytical methods down to better than the femtomole level.

ACKNOWLEDGMENTS

We thank Drs. Anatol Eberhard, Tom Fagan, Pete Greenberg, Margaret McFall-Ngai, Jim Morin, David Morse, Deb Robertson, and Ned Ruby for critical reviews of the text and helpful and enjoyable discussions, and the National Science Foundation for support of research in this area.

Literature Cited

Abu-Soud HM, Clark AC, Francisco WA, Baldwin TO, Raushel FM. 1993. Kinetic destabilization of the hydroperoxy flavin intermediate by site-directed modification of the reactive thiol in bacterial luciferase. *J. Biol. Chem.* 268:7699–706

Aflalo C. 1990. Targeting of cloned firefly luciferase to yeast mitochondria. *Biochemistry* 29:4758–66

Anderson JM, Cormier MJ. 1973. Lumisomes, the cellular site of bioluminescence in coelenterates. *J. Biol. Chem.* 248:2937–43

Bae YM, Hastings JW. 1994. Cloning, sequencing and expression of dinoflagellate luciferase DNA from a marine alga, *Gonyaulax polyedra*. *Biochim. Biophys. Acta* 1219:449–56

Baldwin TO, Ziegler MM. 1992. The biochemistry and molecular biology of bacterial luminescence. In *Chemistry and Biochemistry*

of Flavoenzymes, ed. F Muller, pp. 467–530. Boca Raton: CRC Press

Barros MB, Bechara EJH. 1998. Bioluminescence as a possible auxiliary oxygen detoxifying mechanism in Elaterid larvae. *Free Radic. Biol. Med.* 24:767–77

Bassler BL, Wright M, Showalter RE, Silverman MR. 1993. Intercellular signalling in *Vibrio harveyi*: sequence and function of genes regulating expression of luminescence. *Mol. Microbiol.* 9:773–86

Bassot J-M. 1987. A transient intracellular coupling explains the facilitation of responses in the bioluminescent system of scale-worms. *J. Cell Biol.* 105:2235–43

Bassot J-M, Bilbaut A. 1977. Bioluminescence des elytres d'Acholoe IV. Luminescence et fluorescence des photosomes. *Biol. Cell.* 28:163–68

Bassot J-M, Nicolas M-T. 1978. Similar paracrystals of endoplasmic reticulum in the photoemitters and the photoreceptors of scale worms. *Experientia* 34:726–28

Berzhanskaya LY, Gitelson II, Fish AM, Chumakova RI. 1975. Pulsed nature of bacterial luminescence. *Ser. Biol. Dokl. Akad. Nauk. SSSR* 222:1220–22

Bilbaut A, Bassot J-M. 1977. Bioluminescence des elytres d'Acholoe II. Donnees photometriques. *Biol. Cell.* 28:145–54

Boettcher KJ, Ruby EG. 1995. Detection and quantification of *Vibrio fischeri* autoinducer from symbiotic squid light organs. *J. Bacteriol.* 1995:1053–58

Boettcher KJ, Ruby EG, McFall-Ngai MJ. 1996. Bioluminescence in the symbiotic squid *Euprymna scolopes* is controlled by a daily biological rhythm. *J. Comp. Physiol. A* 179:65–73

Brejc K, Sixma TK, Kitts PA, Kain SR, Tsien RY, et al. 1997. Structural basis for dual excitation and photoisomerization of the *Aequorea victoria* green fluorescent protein. *Proc. Natl. Acad. Sci. USA* 94:2306–11

Case JF, Strause LG. 1978. Neurally controlled luminescent systems. In *Bioluminescence in Action*, ed. PJ Herring, pp. 331–66. London: Academic

Catalani LH, Wilson T. 1989. Electron transfer and chemiluminescence. Two inefficient systems: 1,4-dimethoxy-9,10-diphenylanthracene peroxide and diphenoyl peroxide. *J. Am. Chem. Soc.* 111:2633–39

Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. 1994. Green fluorescent protein as a marker for gene expression. *Science* 263:802–5

Chattoraj M, King BA, Bublitz GU, Boxer SG. 1996. Ultra-fast excited state dynamics in green fluorescent protein: multiple states and proton transfer. *Proc. Natl. Acad. Sci. USA* 93:8362–67

Colepicolo P, Camarero VCCP, Karnovsky ML, Nicolas M-T, Bassot J-M, Hastings JW. 1990. A sensitive and specific assay for superoxide anion released by neutrophils and macrophages, based on polynoidin bioluminescence. *Anal. Biochem.* 184:369–74

Colepicolo P, Cho KW, Poinar GO, Hastings JW. 1989a. Growth and luminescence of the bacterium *Xenorhabdus luminescens* from a human wound. *Appl. Environ. Microbiol.* 55:2601–6

Colepicolo P, Nicolas M-T, Bassot J-M, Hastings JW. 1989b. Expression and localization of bacterial luciferase determined by immunogold labeling. *Arch. Microbiol.* 152: 72–76

Colepicolo P, Roenneberg T, Morse DM, Taylor WR, Hastings JW. 1993. Circadian regulation of bioluminescence in the dinoflagellate *Pyrocystis lunula*. *J. Phycol.* 29:173–79

Conti E, Franks NP, Brick P. 1996. Crystal structure of firefly luciferase throws light on a superfamily of adenylate-forming enzymes. *Structure* 4:287–98

Cormier MJ. 1978. Comparative biochemistry of animal systems. In *Bioluminescence in Action*, ed. PJ Herring, pp. 75–108. London: Academic

Cubitt AB, Heim R, Adams SR, Boyd AE, Gross LA, Tsien RY. 1995. Understanding, improving and using green fluorescent proteins. *Trends Biochem. Sci.* 20:448–55

Cutler MW, Ward WW. 1997. Spectral analysis and proposed model for GFP dimerization. In *Bioluminescence and Chemiluminescence*, ed. JW Hastings, LJ Kricka, PE Stanley, pp. 403–6. Chichester: Wiley & Sons

Daubner SC, Astorga AM, Leisman GB, Baldwin TO. 1987. Yellow light emission of *Vibrio fischeri* strain Y-1: purification and characterization of the energy-accepting yellow fluorescent protein. *Proc. Natl. Acad. Sci. USA* 84:8912–16

DeLuca M. 1969. Hydrophobic nature of the active site of firefly luciferase. *Biochemistry* 8:160–66

Desjardins M, Morse D. 1993. The polypeptide components of scintillons, the bioluminescence organelles of the dinoflagellate *Gonyaulax polyedra*. *Biochem. Cell Biol.* 71:176–82

Devine JH, Kutuzova GD, Green VA, Ugarova NN, Baldwin TO. 1993. Luciferase from the East European firefly *Luciola mingrelica*: cloning and nucleotide sequence of the cDNA, overexpression in *Escherichia coli* and purification of the enzyme. *Biochim. Biophys. Acta* 1173:121–32

De Wet JR, Wood KV, DeLuca M, Helinski DR,

Subramani S. 1987. Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell. Biol.* 7:725–37

Dunlap K, Takeda K, Brehm P. 1987. Activation of a calcium-dependent photoprotein by chemical signalling through gap junctions. *Nature* 325:60–62

Dunlap PV. 1992. Mechanism for iron control of the *Vibrio fischeri* luminescence system: involvement of cyclic AMP and cyclic AMP receptor protein and modulation of DNA level. *J. Biolum. Chemilum.* 7:203–14

Dunphy G, Miyamoto C, Meighen E. 1997. A homoserine lactone autoinducer regulates virulence of an insect-pathogenic bacterium, *Xenorhabdus nematophilus* (Enterobacteriaceae). *J. Bacteriol.* 179:5288–91

Eakin RM, Brandenburger JL. 1975. Retinal differences between light-tolerant and light avoiding slugs. *J. Ultrastruct. Res.* 53:382–94

Eberhard A, Burlingame AL, Eberhard C, Kenyon GL, Nealson KH, Oppenheimer NJ. 1981. Structural identification of autoinducer of *Photobacterium fischeri* luciferase. *Biochemistry* 20:2444–49

Eberhard A, Longin T, Widrig CA, Stranick SJ. 1991. Synthesis of the *lux* gene autoinducer in *Vibrio fischeri* is positively autoregulated. *Arch. Microbiol.* 155:294–97

Eckert R. 1965. Bioelectric control of bioluminescence in the dinoflagellate *Noctiluca*. *Science* 147:1140–45

Eckstein JW, Cho KW, Colepicolo P, Ghisla S, Hastings JW, Wilson T. 1990. A time-dependent bacterial bioluminescence emission spectrum in an in vitro single turnover system: Energy transfer alone cannot account for the yellow emission of *Vibrio fischeri Y-1*. *Proc. Natl. Acad. Sci. USA* 87: 1466–70

Eckstein JW, Hastings JW, Ghisla S. 1993. Mechanism of bacterial bioluminescence: 4a,5-dihydroflavin analogs as models for luciferase hydroperoxide intermediates and the effect of substituents at the 8-position of flavin on luciferase kinetics. *Biochemistry* 32:404–11

Fisher AJ, Raushel FM, Baldwin TO, Rayment L. 1995. Three-dimensional structure of bacterial luciferase from *Vibrio harveyi* at 2.4 Å resolution. *Biochemistry* 34:6581–86

Fisher AJ, Thompson TB, Thoden JB, Baldwin TO, Rayment I. 1996. The 1.5-Å resolution crystal structure of bacterial luciferase in low salt conditions. *J. Biol. Chem.* 271:21956–68

Forst S, Nealson KH. 1996. Molecular biology of the symbiotic-pathogenic bacteria *Xenorhabdus* spp. and *Photorhabdus* spp. *Microbiol. Rev.* 60:21–43

Fritz L, Morse D, Hastings JW. 1990. The circadian bioluminescence rhythm of *Gonyaulax* is related to daily variations in the number of light emitting organelles. *J. Cell Sci.* 95:321–28

Fuqua C, Winans SC, Greenberg EP. 1996. Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. *Annu. Rev. Microbiol.* 50:727–51

Ghiradella H. 1977. Fine structure of the tracheoles of the lantern of a photurid firefly. *J. Morphol.* 153:187–204

Gonzalez-Flecha B, Demple B. 1994. Intracellular generation of superoxide as a byproduct of *Vibrio harveyi* luciferase expressed in *Escherichia coli*. *J. Bacteriol.* 176:2293–99

Gould SJ, Keller G-A, Hosken N, Wilkinson J, Subramani S. 1989. A conserved tripeptide sorts proteins to peroxisomes. *J. Cell Biol.* 108:1657–64

Haas E. 1980. Bioluminescence from single bacterial cells exhibits no oscillation. *Biophys. J.* 31:301–12

Hanna CH, Hopkins TA, Buck J. 1976. Peroxisomes of the firefly lantern. *J. Ultrastruct. Res.* 57:150–62

Hanzelka BL, Greenberg EP. 1995. Evidence that the N-terminal region of the *Vibrio fischeri* LuxR protein constitutes an autoinducer-binding domain. *J. Bacteriol.* 177: 815–17

Hanzelka BL, Greenberg EP. 1996. Quorum sensing in *Vibrio fischeri*: evidence that *S*-adenosylmethionine is the amino acid substrate for autoinducer synthesis. *J. Bacteriol.* 178:5291–94

Hart RC, Stempel KE, Boyer PD, Cormier MJ. 1978. Mechanism of the enzyme-catalyzed bioluminescent oxidation of coelenterate-type luciferin. *Biochem. Biophys. Res. Commun.* 81:980–86

Hastings JW. 1968. Bioluminescence. *Annu. Rev. Biochem.* 37:597–630

Hastings JW. 1978. Bacterial and dinoflagellate luminescent systems. In *Bioluminescence in Action*, ed. PJ Herring, pp. 129–170. London: Academic

Hastings JW. 1983. Biological diversity, chemical mechanisms, and the evolutionary origins of bioluminescent systems. *J. Mol. Evol.* 19:309–21

Hastings JW, Balny C, Le Peuch C, Douzou P. 1973. Spectral properties of an oxygenated luciferase-flavin intermediate isolated by low-temperature chromatography. *Proc. Natl. Acad. Sci. USA* 70:3468–72

Hastings JW, Dunlap JC. 1986. Cell-free components in dinoflagellate bioluminescence. The particulate activity: scintillons; the soluble components: luciferase, luciferin,

and luciferin-binding protein. *Meth. Enzymol.* 133:307–23

Hastings JW, Morin JG. 1991. Bioluminescence. In *Neural and Integrative Animal Physiology*, ed. CL Prosser, pp. 131–70. New York: Wiley Intersci.

Heim R, Prasher DC, Tsien RY. 1994. Wavelength mutations and posttranslational autoxidation of green fluorescent protein. *Proc. Natl. Acad. Sci. USA* 91:12501–4

Heim R, Tsien RY. 1996. Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. *Curr. Biol.* 6:178–82

Herring PJ, ed. 1978. *Bioluminescence in Action*. London: Academic. 570 pp.

Hosseini PK, Nealson KH. 1995. Symbiotic luminous soil bacteria: unusual regulation for an unusual niche. *Photochem. Photobiol.* 62:633–40

Inouye S, Ohmiya Y, Toya Y, Tsuji FI. 1992. Imaging of luciferase secretion from transformed Chinese hamster ovary cells. *Proc. Natl. Acad. Sci. USA* 89:9584–87

Johnson CH, Hastings JW. 1986. The elusive mechanism of the circadian clock. *Am. Sci.* 74:29–36

Johnson CH, Inoue S, Flint A, Hastings JW. 1985. Compartmentalization of algal bioluminescence: autofluorescence of bioluminescent particles in the dinoflagellate *Gonyaulax* as studied with image-intensified video microscopy and flow cytometry. *J. Cell Biol.* 100:1435–46

Johnson CH, Knight MR, Kondo T, Masson P, Sedbrook J, et al. 1995. Circadian oscillations of cytosolic and chloroplastic free calcium in plants. *Science* 259:1863–65

Johnson FH, Shimomura O, Saiga Y, Gershman G, Reynolds GT, Waters JR. 1962. Quantum efficiency of *Cypridina* luminescence with a note on that of *Aequorea*. *J. Cell. Comp. Physiol.* 60:85–103

Kajiyama N, Nakano E. 1991. Isolation and characterization of mutants of firefly luciferase which produce different colors of light. *Protein Eng.* 4:691–93

Karatani H, Hastings JW. 1993. Two active forms of the accessory yellow fluorescence protein of the luminous bacterium *Vibrio fischeri* strain Y1. *Photochem. Photobiol.* 18:227–32

Keller G-A, Gould SJ, DeLuca M, Subramani S. 1987. Firefly luciferase is targeted to peroxisomes in mammalian cells. *Proc. Natl. Acad. Sci. USA* 84:3264–68

Khan AU, Wilson T. 1995. Reactive oxygen species as cellular messengers. *Chem. Biol.* 2:437–45

Knaust R, Urbig T, Li L, Taylor W, Hastings JW. 1998. The circadian rhythm of bioluminescence in *Pyrocystis* is not due to differences in the amount of luciferase: a comparative study of three bioluminescent marine dinoflagellates. *J. Phycol.* 34:167–72

Koka P, Lee J. 1979. Separation and structure of the prosthetic group of the blue fluorescence protein from the bioluminescent bacterium *Photobacterium phosphoreum*. *Proc. Natl. Acad. Sci. USA* 76:3068

Koo J-Y, Schmidt SP, Schuster GB. 1978. Bioluminescence of the firefly: key steps in the formation of the electronically excited state for model systems. *Proc. Natl. Acad. Sci. USA* 75:30–33

Kurfürst M, Macheroux P, Ghisla S, Hastings JW. 1987. Isolation and characterization of the transient, luciferase-bound flavin-4a-hydroxide in the bacterial luciferase reaction. *Biochim. Biophys. Acta* 924:104–10

Lamola AA. 1969. *Energy Transfer and Organic Photochemistry*. New York: Interscience. 374 pp.

Lee D-H, Mittag M, Sczekan S, Morse D, Hastings JW. 1993. Molecular cloning and genomic organization of a gene for luciferin-binding protein from the dinoflagellate *Gonyaulax polyedra*. *J. Biol. Chem.* 268:8842–50

Lee J, Matheson IBC, Muller F, O'Kane DJ, Vervoort J, Visser AJWG. 1991. The mechanism of bacterial bioluminescence. In *Chemistry and Biochemistry of Flavoenzymes*, ed. F Muller, pp. 109–51. Orlando: CRC Press

Lee K-H, Ruby EG. 1994. Competition between *Vibrio fischeri* strains during initiation and maintenance of a light organ symbiosis. *J. Bacteriol.* 176:1985–91

Li L, Hastings JW. 1998. The structure and organization of the luciferase gene in the photosynthetic dinoflagellate *Gonyaulax polyedra*. *Plant Mol. Biol.* 36:275–84

Li L, Hong R, Hastings JW. 1997. Three functional luciferase domains in a single polypeptide chain. *Proc. Natl. Acad. Sci. USA* 94:8954–58

Lorenz WW, Cormier MJ, O'Kane DJ, Hua D, Escher AA, Szalay AA. 1996. Expression of the *Renilla reniformis* luciferase gene in mammalian cells. *J. Biolumin. Chemilumin.* 11:31–37

Lorenz WW, McCann RO, Longiaru M, Cormier MJ. 1991. Isolation and expression of a cDNA encoding *Renilla reniformis* luciferase. *Proc. Natl. Acad. Sci. USA* 88:4438–42

Macheroux P, Ghisla S, Hastings JW. 1993. Spectral detection of an intermediate preceding the excited state in the bacterial luciferase reaction. *Biochemistry* 32:14183–86

Macheroux P, Schmidt KU, Steinerstauch P,

Ghisla S, Colepicolo P, et al. 1987. Purification of the yellow fluorescent protein from *Vibrio fischeri* and identity of the flavin chromophore. *Biochem. Biophys. Res. Commun.* 146:101–6

Mager IX, Tu S-C. 1995. Chemical aspects of bioluminescence. *Photochem. Photobiol.* 62:607–14

Masuda T, Tatsumi H, Nakano E. 1989. Cloning and sequence analysis of cDNA for luciferase of a Japanese firefly, *Luciola cruciata*. *Gene* 77:265–70

Mayerhofer R, Langridge WHR, Cormier MJ, Szalay AA. 1995. Expression of recombinant *Renilla* luciferase in transgenic plants results in high levels of light emission. *Plant J.* 7:1031–38

McCapra F. 1997. Mechanisms in chemiluminescence and bioluminescence—unfinished business. In *Bioluminescence and Chemiluminescence*, ed. JW Hastings, LJ Kricka, PE Stanley, pp. 7–15. Chichester: Wiley & Sons

McCapra F, Gilfoyle DJ, Young DW, Church NJ, Spencer P. 1994. The chemical origin of colour differences in beetle bioluminescence. In *Bioluminescence and Chemiluminescence*, ed. AK Campbell, LJ Kricka, PE Stanley, pp. 387–91. Chichester: Wiley & Sons

McCapra FM, Perring KD. 1985. Luciferin bioluminescence. In *Chemi- and Bioluminescence*, ed. JG Burr, pp. 359–86. New York: Dekker

McElroy WD, DeLuca M. 1985. Firefly luminescence. In *Chemi- and Bioluminescence*, ed. JG Burr, pp. 387–99. New York: Dekker

McElroy WD, Seliger HH. 1962. Origin and evolution of bioluminescence. In *Horizons in Biochemistry*, ed. M Kasha & B Pullman, pp. 91–101. New York: Academic

McFall-Ngai MJ, Ruby EG. 1991. Symbiont recognition and subsequent morphogenesis as early events in an animal-bacterial mutualism. *Science* 254:1491–94

Meighen EA. 1994. Genetics of bacterial bioluminescence. *Annu. Rev. Genet.* 28:117–39

Merenyi G, Lind J, Mager HIX, Tu S-C. 1992. Properties of 4a-hydroxy-4a,5-dihydroflavin radicals in relation to bacterial bioluminescence. *J. Phys. Chem.* 96:10528–33

Mitra RD, Silva CM, Youvan DC. 1996. Fluorescence resonance energy transfer between blue-emitting and red-shifted excitation derivatives of the green fluorescent protein. *Gene* 173:13–17

Mittag M, Eckerskorn C, Strupat K, Hastings JW. 1997. Differential translational initiation of *lbp* mRNA is caused by a small 5′ open reading frame. *FEBS Lett.* 411:245–50

Mittag M, Lee D-H, Hastings JW. 1994. Circadian expression of the luciferin-binding protein correlates with the binding of a protein to its 3′ untranslated region. *Proc. Natl. Acad. Sci. USA* 91:5257–61

Mittag M, Li L, Hastings JW. 1998. The mRNA level of the circadian regulated *Gonyaulax* luciferase remains constant over the cycle. *Chronobiol. Int.* 15:93–98

Montgomery MK, McFall-Ngai MJ. 1994. Bacterial symbionts induce host organ morphogenesis during early postembryonic development of the squid *Euprymna scolopes*. *Development* 120:1719–29

Moré MI, Finger LD, Stryker JL, Fuqua C, Eberhard A, Winans SC. 1996. Enzymatic synthesis of a quorum-sensing autoinducer through use of defined substrates. *Science* 272:1655–58

Morin JG, Hastings JW. 1971. Energy transfer in a bioluminescent system. *J. Cell. Physiol.* 77:313–18

Morse D, Milos PM, Roux E, Hastings JW. 1989a. Circadian regulation of bioluminescence in *Gonyaulax* involves translational control. *Proc. Natl. Acad. Sci. USA* 86:172–76

Morse D, Pappenheimer AM, Hastings JW. 1989b. Role of a luciferin binding protein in the circadian bioluminescent reaction of *G. polyedra*. *J. Biol. Chem.* 264:11822–26

Nakamura H, Kishi Y, Shimomura O, Morse D, Hastings JW. 1989. Structure of dinoflagellate luciferin and its enzymatic and nonenzymatic air-oxidation products. *J. Am. Chem. Soc.* 111:7607–11

Nealson KH, Platt T, Hastings JW. 1970. The cellular control of the synthesis and activity of the bacterial luminescent system. *J. Bacteriol.* 104:313–22

Nicolas M-T, Bassot J-M, Shimomura O. 1982. Polynoidin: a membrane photoprotein isolated from the bioluminescent system of scale-worms. *Photochem. Photobiol.* 35:201–7

Nicolas M-T, Morse D, Bassot J-M, Hastings JW. 1991. Colocalization of luciferin binding protein and luciferase to the scintillons of *Gonyaulax polyedra* revealed by double immunolabeling after fast-freeze fixation. *Protoplasma* 160:159–66

Nicolas M-T, Nicolas G, Johnson CH, Bassot J-M, Hastings JW. 1987a. Characterization of the bioluminescent organelles in *Gonyaulax polyedra* (dinoflagellates) after fast-freeze fixation and antiluciferase immunogold staining. *J. Cell Biol.* 105:723–35

Nicolas M-T, Sweeney BM, Hastings JW. 1987b. The ultrastructural localization of luciferase in three bioluminescent dinoflagellates, two species of *Pyrocystis*, and *Noctiluca*, using anti-luciferase and immunogold labeling. *J. Cell Sci.* 87:189–96

O'Kane DJ, Prasher DC. 1992. Evolutionary

origins of bacterial bioluminescence. *Mol. Microbiol.* 6:443–49

O'Kane DJ, Woodward B, Lee J, Prasher DC. 1991. Borrowed proteins in bacterial bioluminescence. *Proc. Natl. Acad. Sci. USA* 88:1100–4

Ormö M, Cubitt AB, Kallio K, Gross LA, Tsien RY, Remington SJ. 1996. Crystal structure of the Aequorea victoria green fluorescent protein. *Science* 273:1392–95

Prasher DC, Eckenrode VK, Ward WW, Prendergast FG, Cormier MJ. 1992. Primary structure of the *Aequorea victoria* green-fluorescent protein. *Gene* 111:292–93

Petushkov VN, Gibson BG, Lee J. 1996a. Direct measure of excitation transfer in the protein complex of bacterial luciferase hydroxyflavin and the associated yellow fluorescence proteins from *Vibrio fischeri* Y1. *Biochemistry* 35:8413–18

Petushkov VN, Ketelaars M, Gibson BG, Lee J. 1996b. Interaction of *Photobacterium leiognathi* and *Vibrio fischeri* Y1 luciferases with fluorescent (antenna) proteins: bioluminescence effects of the aliphatic additive. *Biochemistry* 35:12086–93

Puskas A, Greenberg EP, Kaplan S, Schaefer AL. 1997. A quorum-sensing system in the free-living photosynthetic bacterium *Rhodobacter sphaeroides*. *J. Bacteriol.* 179:7530–37

Rees J-F, De Wergifosse B, Noiset O, Dubuisson M, Janssens B, Thompson EM. 1998. The origins of marine bioluminescence: turning oxygen defence mechanisms into deep-sea communication tools. *J. Exp. Biol.* 201:1211–21

Reich KA, Schoolnik GK. 1996. Halovibrin, secreted from the light organ symbiont *Vibrio fischeri*, is a member of a new class of ADP-ribosyltransferases. *J. Bacteriol.* 178:209–15

Reid BG, Flynn GC. 1997. Chromophore formation in green fluorescent protein. *Biochemistry* 36:6786–91

Ruby EG. 1996. Lessons from a cooperative, bacterial-animal association: the *Vibrio fischeri—Euprymna scolopes* light organ symbiosis. *Annu. Rev. Microbiol.* 50:591–624

Ruby EG, Asato LM. 1993. Growth and flagellation of *Vibrio fischeri* during initiation of the sepiolid squid light organ symbiosis. *Arch. Microbiol.* 159:160–67

Ruby EG, McFall-Ngai MJ. 1992. A squid that glows in the night: development of an animal-bacterial mutualism. *J. Bacteriol.* 174:4865–70

Schaefer AL, Val DL, Hanzelka BL, Cronan JE, Greenberg EP. 1996. Generation of cell-to-cell signals in quorum sensing: acyl homoserine lactone synthase activity of a purified *Vibrio fischeri* LuxI protein. *Proc. Natl. Acad. Sci. USA* 93:9505–9

Schmitter RE, Njus D, Sulzman FM, Gooch VD, Hastings JW. 1976. Dinoflagellate bioluminescence: a comparative study of in vitro components. *J. Cell. Physiol.* 87:123–34

Schroder J. 1989. Protein sequence homology between plant 4-coumarate:CoA ligase and firefly luciferase. *Nucleic Acids Res.* 17:460

Schuster GB. 1979. Chemiluminescence of organic peroxides. Conversion of ground-state reactants to excited-state products by the chemically initiated electron-exchange luminescence mechanism. *Acc. Chem. Res.* 12:366–73

Seliger HH. 1975. The origin of bioluminescence. *Photochem. Photobiol.* 21:355–61

Seliger HH. 1987. The evolution of bioluminescence in bacteria. *Photochem. Photobiol.* 45:291–97

Shimomura O. 1982. Mechanism of bioluminescence. In *Chemical and Biological Generation of Excited States*, ed. W Adam, G Cilento, pp. 249–76. New York: Academic

Shimomura O. 1987. Presence of coelenterazine in non-bioluminescent marine organisms. *Comp. Biochem. Physiol.* 86B:361–63

Shimomura O, Inoue S, Johnson FH, Haneda Y. 1980. Widespread occurrence of coelenterazine in marine bioluminescence. *Comp. Biochem. Physiol.* 65B:435–37

Shimomura O, Johnson FH, Saiga Y. 1962. Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, *Aequorea*. *J. Cell. Comp. Physiol.* 59:223–40

Shimomura O, Satoh S, Kishi Y. 1993. Structure and non-enzymatic light emission of two luciferin precursors isolated from the luminous mushroom *Panellus-stipticus*. *J. Biolum. Chemilum.* 8:201–5

Sirokmán G, Hastings JW. 1997. Effectiveness of the accessory yellow fluorescent protein in the bacterial luciferase reaction correlates with the lifetime of the peroxyhemiacetal intermediate: the stereochemistry of the reaction. *Photochem. Photobiol.* 66:198–203

Sirokmán G, Wilson T, Hastings JW. 1995. A bacterial luciferase reaction with a negative temperature coefficient attributable to protein-protein interaction. *Biochemistry* 34:13074–81

Sitnikov DM, Schineller JB, Baldwin TO. 1995. Transcriptional regulation of bioluminescence genes from *Vibrio fischeri*. *J. Bacteriol.* 17:801–12

Soto U, Pepperkok R, Ansorge W, Just WW. 1993. Import of firefly luciferase into mammalian peroxisomes in vivo requires nucle-

oside triphosphates. *Exp. Cell Res.* 205:66–75

Spurlock BO, Cormier MJ. 1975. A fine structure study of the anthocodium in Renilla mülleri. Evidence for the existence of a bioluminescent organelle, the luminelle. *J. Cell. Biol.* 64:15–28

Steinbeck MJ, Khan AU, Karnovsky MJ. 1992. Intracellular singlet oxygen generation by phagocytosing neutrophils in response to particles coated with a chemical trap. *J. Biol. Chem.* 267:13425–33

Stevens AM, Greenberg EP. 1997. Quorum sensing in *Vibrio fischeri:* essential elements for activation of the luminescence genes. *J. Bacteriol.* 179:557–62

Swift S, Throup JP, Williams P, Salmond GPC, Stewart GSAB. 1996. Quorum sensing: a population-density component in the determination of bacterial phenotype. *Trends Biochem. Sci.* 21:214–19

Thomson CM, Herring PJ, Campbell AK. 1997. The widespread occurrence and tissue distribution of the imidazolopyrazine luciferins. *J. Biolum. Chemilum.* 12:87–91

Tsuji FI, Ohmiya Y, Fagan TF, Toh H, Inouye S. 1995. Molecular evolution of the Ca^{2+}-binding photoproteins of the Hydrozoa. *Photochem. Photobiol.* 62:657–61

Tu S-C, Mager HIX. 1995. Biochemistry of bacterial bioluminescence. *Photochem. Photobiol.* 62:615–24

Turro NJ. 1978. *Modern Molecular Photochemistry.* Menlo Park: Benjamin/Cummings. 628 pp.

Ulitzur S, Dunlap PV. 1995. Regulatory circuitry controlling luminescence autoinduction in *Vibrio fischeri. Photochem. Photobiol.* 62:625–32

Vervoort J, Muller F, Lee J, van den Berg WAM, Moonen CTW. 1986. Identification of the true carbon-13 nuclear magnetic resonance spectrum of the stable intermediate II in bacterial luciferase. *Biochemistry* 25:8062–67

Viviani VR, Bechara EJH. 1997. Bioluminescence and biological aspects of Brazilian railroad worms (Coleoptera: Phengodidae). *Ann. Entomol. Soc. Am.* 90:389–98

Wachter RM, King BA, Heim R, Kallio K, Tsien RY, et al. 1997. Crystal structure and photodynamic behavior of the blue emission variant Y66H/Y145F of green fluorescent protein. *Biochemistry* 36:9759–66

Ward WW, Cormier MJ. 1976. In vitro energy transfer in *Renilla* bioluminescence. *J. Phys. Chem.* 80:2289–91

Ward WW, Cormier MJ. 1978. Energy transfer via protein-protein interaction in *Renilla* bioluminescence. *Photochem. Photobiol.* 27:389–96

Weis VM, Small AL, McFall-Ngai MJ. 1996. A peroxidase related to the mammalian antimicrobial protein myeloperoxidase in the *Euprymna-Vibrio* mutualism. *Proc. Natl. Acad. Sci. USA* 93:13683–88

White EH, Roswell DF. 1991. Analogs and derivatives of firefly oxyluciferin, the light emitter in firefly bioluminescence. *Photochem. Photobiol.* 53:131–36

Widder EA, Case JF. 1982. Distribution of subcellular bioluminescence sources in a dinoflagellate, *Pyrocystis fusiformis. Biol. Bull.* 162:423–48

Wilson T. 1995. Comments on the mechanisms of chemi- and bioluminescence. *Photochem. Photobiol.* 62:601–6

Wood KV. 1995. The chemical mechanism and evolutionary development of beetle bioluminescence. *Photochem. Photobiol.* 62:662–73

Wood KV, Lam YA, Seliger HH, McElroy WD. 1989. Complementary DNA coding click beetle luciferases can elicit bioluminescence of different colors. *Science* 244:700–2

Wu P, Brand L. 1994. Resonance energy transfer: methods and applications. *Analyt. Biochem.* 218:1–13

Yang F, Moss LG, Phillips GN Jr. 1996. The molecular structure of green fluorescent protein. *Nat. Biotechnol.* 14:1246–51

Annual Review of Cell and Developmental Biology
Volume 14, 1998

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